Method for the early diagnosis of carcinomas, and test kit for implementing it

The present invention relates to a method for the early diagnosis of carcinomas, or preliminary stages thereof, which are caused by human papilloma viruses or are associated with human papilloma viruses. The invention also relates to a test kit for implementing the method.

Screening programs for different carcinomas have been available in Germany since the end of the 1960s. In the case of cervical carcinoma, these programs are based on subjecting cell smears taken from the cervix uteri to a cytomorphologic examination in accordance with the method of G. Papanicolaou, i.e. what is termed the Pap test. In the case of the Pap test, cell material is taken from women at regular intervals within the context of the routine gynecological examination and applied to a glass slide and stained. The smears are classified on the basis of the morphology of the cells, being designated a smear without pathological findings, an inflammatory change, slight dysplasia, moderate dysplasia, severe dysplasia, carcinoma in situ, or carcinoma corresponding to Pap I to V (see H.J. Soost, The Munich nomenclature, *Recent Results Cancer Res.* **133** [1993] pp. 105-111). If the result of the Pap test indicates a pathological change, a biopsy is taken and subjected to a histopathologic examination, which establishes the nature and extent of the dysplasia and categorizes it as cervical intraepithelial neoplasia (CIN I to III). The use of the Pap test over the last 30 years has shown that, within a group suffering from equally severe malignant degeneration, the probability of a remission leading to recovery decreases as the severity of the dysplasia increases.

It has not thus far been possible to make any predictions with regard to remission or progression behavior in the case of individual preparations.

Papilloma viruses are small double-stranded DNA viruses having a genome of approx. 8000 base pairs. At present, more than 150 different virus types which are pathogenic to humans are known. In accordance with their epithelial tropism, the papilloma viruses can be assigned either to the mucocutaneous group (wart formation and kutaneous neoplasias) or to the anogenital group (condylomas and anal and cervical carcinomas).

In most cases, infection with HPV is asymptomatic. For this reason, the latent virus infection has to be distinguished from the viral disease involving tissue lesions which can be discerned microscopically ("subclinically") or clinically.

Some types of papilloma virus are carcinogenic, i.e. these virus types (e.g. HPV 16 and 18) can contribute crucially to the transformation of epithelial cells. These viruses are described as the high-risk group. This group is in contrast to the low-risk group of virus types (e.g. 6 and 11) which are either not carcinogenic or only very slightly carcinogenic.

HPV infection is the most frequent sexually transmissible infection in humans. The data on prevalence differ considerably and depend on the source clientele being examined. Following assumption of the first sexual contacts, the rate of infection initially increases drastically in a natural manner. In North America and parts of Europe, the degree of contagion with human papilloma viruses in the 15 to 25-year-old

age group of women is greater than 50 %. The rate of detected infections falls continuously with increasing age. It is less than 5 % after the 50th year of life.

The removal of cervical cells, with the cells then being subjected to a Papanicolaou staining, is carried out as a screening method when women undergo a medical check-up. This method makes possible the early detection of changes in cell morphology (preliminary stages of cancer) which occur during the development of cervical carcinoma. These routinely performed examinations have contributed to the incidence of cervical carcinoma falling continuously. Nevertheless, about 6000 women contract cervical carcinoma for different reasons in Germany every year.

Human papilloma viruses play a crucial role in the development of cervical carcinoma. For this reason, supporting diagnostic methods are directed towards detecting infection with the virus. A large number of latent infections are detected when molecular biological methods such as PCR or nucleic acid hybridization are used to detect the viral nucleic acid, owing to the extremely high sensitivity of these methods. The detection of DNA, which is always positive when an HPV infection is present, is consequently independent of the course of the productive phase and does not, therefore, permit any conclusion with regard to prognosis. The importance of viral protein synthesis for the course of the infection, and the prognostic importance which results from this, have not thus far been investigated.

It is known that the following proteins are formed in dependence on the viral life cycle: early protein (E)1, E2, E4, E5, E6 and E7, and late protein (L)1 and L2

(H.R. McMurray, D. Nguyen, T.F. Westbrook, D.J. Mc Cance, Biology of human papilloma viruses, in *Int. J. Exp. Pathol.* **82** [2001] 15 - 33).

The capsid protein L1 is synthesized in the cytoplasma of the host cell and then transported into the nucleus of the cell. In the nucleus, the capsid protein interacts with the viral DNA and forms mature viruses. Detecting the capsid protein L1 therefore makes it possible to conclude that the virus is passing through a productive phase. Mature, infectious viruses are released by the infected cell. In one particular embodiment, the method is based on monoclonal antibodies which specifically recognize the papilloma virus capsid protein L1. Suitable monoclonal antibodies are disclosed in DE 43 32 596 A1. The screening test recognizes all papilloma viruses which are thus far known and is suitable, therefore, for detecting whether the capsid antigen is being formed and whether an HPV infection is in its productive phase or not. The high-risk detection selectively recognizes the HPV risk types 16, 18, 33, 35, 39, 45, 51, 56 and 58 (see, for example, M. Sapp, U. Kraus, C. Volpers, P.J.F. Snijders, J.M.M. Walboomers, J.E. Streeck, Analysis of type-restricted and cross-reactive epitopes on virus-like particles of human papilloma virus type 33 and in infected tissue using monoclonal antibodies to the major capsid protein, in *J. Gen. Virol.* **75** [1994] 3375 - 3383).

In addition, L1-specific antibodies are available for detecting individual HPV types, e.g. HPV 16.

The present invention is based on the observation that, in the case of slight to moderate dysplasias which are high-risk HPV DNA-positive, the formation of the human

papilloma virus capsid antigen L1 is associated with a regression of the malignant degeneration whereas the absence of HPV envelope proteins correlates with the disease progressing. The invention therefore makes it possible to make a relatively viable prognosis as to whether a malignant degeneration will develop.

The invention accordingly relates to a method using body samples for the early diagnosis of carcinomas, or preliminary stages thereof, which are caused by human papilloma viruses or are associated with human papilloma viruses, characterized in that it is established whether a capsid protein of a human papilloma virus (HPV) is being expressed.

The expression "carcinomas and preliminary stages thereof" encompasses carcinomas of any type and origin, as well as preliminary stages thereof, which are associated with human papilloma viruses. For example, the carcinomas can be carcinomas of the anogenital tract, in particular cervical carcinoma. The preliminary stages, e.g. slight to moderate dysplasia (cervical intraepithelial neoplasia (CIN I - III), etc., are also to be mentioned, in particular.

The expression "capsid protein of the human papilloma viruses" refers to the capsid proteins of all HPV types, in particular the (major) capsid protein L1 and the (minor) capsid protein L2 of the viruses.

The expression "body sample" encompasses any body sample in which the capsid protein can be detected. Examples of such body samples are smears, biopsies, organ puncture fluids, blood, sputum, urine, feces, cerebrospinal fluid, lymph fluid, etc. In particular

smears and biopsies are meant when it is a matter of detecting cervical carcinoma.

The expression "determining capsid formation" encompasses all methods which are suitable for detecting the formation of the capsid antigen, its mRNA, its intermediates or its precursors. The expression also explicitly means transcription factors which exert an influence on, and/or control, the formation of the capsid protein as well as serological and/or immunological reactions which are to be attributed to the capsid antigen.

The expression of the capsid protein can be detected at the nucleic acid level and/or at the protein level.

Immunohistochemical or immunocytochemical staining methods, and also western blotting, ELISA or immunoprecipitation, can, for example, be used for detection at the protein level. It is also possible to use antibodies which are directed against the capsid antigen. It may be advantageous for the antibodies to be fixed on solid supports such as microtiter plates, test strips or latex particles.

The present method can be used to diagnose the course of a carcinoma disease at an early stage, i.e. in its preliminary stages.

The invention furthermore relates to a test kit for detecting carcinomas, or preliminary stages thereof, which kit comprises a reagent which can be used to establish whether an HPV capsid protein has been expressed.

In this connection, the capsid protein functions in a general manner as an antigen. The test kit preferably comprises, as detection reagent, an anti-mouse immunoglobulin in combination with an enzyme, preferably a peroxidase. The test kit also comprises a substance, expediently a chromogen solution, for staining the preparation and can, in addition to this, contain customary constituents such as buffers, supports and/or labels.

The present invention can be used to diagnose carcinomas at an early stage. In particular, preliminary stages of carcinomas can be recognized at an early stage and the courses of the disease can be determined.

Another characteristic feature is that the results which are achieved using the method according to the invention are not assessed subjectively. Furthermore, the present invention can be performed rapidly and readily, thereby making it suitable for large-scale screening measures, in particular in third-world countries. The present invention therefore represents an important contribution to the modern diagnosis of HPV-associated cancer diseases.

The invention is explained by means of the following example:

A group of 86 cytological smears, all of which exhibited slight to moderate dysplasia and all of which were high-risk HPV DNA-positive, were examined for the formation of the capsid antigen L1 using L1-specific antibodies. The examination was carried out using the ®Viroactiv kit (obtainable from Virofem Diagnostik und Forschungs [diagnosis and research] GmbH), which

comprises antibodies directed against the capsid protein L1. The preparations were boiled and incubated at room temperature for 30 min after the antibodies had been added. The positive cell nuclei become stained as a result of the consecutive addition of the detection reagent (goat anti-mouse immunoglobulin) and the chromogen (AEC) solution.

A smear was judged to be positive when the nucleus of at least one cell in the specimen exhibited a specific red staining.

On this criterion, 29 specimens were positive, with this corresponding to 33.7 %. The remission frequency was 77.3 % in the women aged over 25 in whom the capsid antigen was formed, while it was as much as 82.4 % in the women aged over 30. The probability of progression to histologically established severe dysplasias or carcinoma in situ was only 22.7 % and 17.6 %, respectively.

57 specimens were negative for the formation of the capsid antigen. This corresponds to 66.2 %. A remission was only seen in 25 % of the women aged over 25 in whom the capsid antigen was not detectable, while progression was seen in 75 %. In the women aged over 30, the remission frequency was 22.2 %. The condition progressed in the remaining 77.8 %.